Res. on Crops **21** (3) : 650-657 (2020) DOI : 10.31830/2348-7542.2020.101
With four figures and one plate
Printed in India

High-resolution GC/MS study of biodegradation of crude oil by *Bacillus megaterium*

MARWAH T. ALNUAIMI[1], TARA A. TAHER[2], ZAHRAA ZAHRAW ALJANABI[3] AND MANAL M. ADEL[4,*]

[1]*Department of Environment Technology and Water Technology Researches*
Division of Pollution Treatment Technology, Ministry of Science and Technology
Baghdad, Iraq
**(e-mail : mhassanein11@hotmail.com)*

(Received : June 23, 2020/Accepted : August 20, 2020)

ABSTRACT

Bacillus megaterium believed to be capable of crude oil degradation through the formation of the pure crude oil emulsion layer. This study was conducted during March 2019 to January 2020 at the Directorate of Environment and Water, Baghdad, Iraq to evaluate the bacterial biodegradation activity using Gas-Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). The visual GC-MS examination confirmed the disappearing trend of several chemicals, as well as decrease in the peak area for other components, indicating the efficiency of the bacteria in the oil decomposition and dismantling of hydrocarbons. The GC-MS analysis of crude oil treatment showed complete mineralization into low molecular weight compounds as Butanoic acid, 1-1-dimethylethyl ester, Benz [c] pyran-1,3-dione, 4,4-dimethyl, 1,2-Benezenedicarboxylic acid, diisooctyl ester, etc. Therefore, *Bacillus megaterium* can be effectively utilized for biodegradation of crude oil-contaminated soil and water ecosystems.

Key words : *Bacillus megaterium*, biodegradation, crude oil, gas-chromatography, pollution

INTRODUCTION

Oil spills or hydrocarbon contamination remains a major environmental problem today, due to the activities of the related industries, such as the petrochemical industry, during the industrial process, oil spills are normally discharged into water bodies, causing serious problems to the ecosystem (Misra and Pandey, 2005; Rajput *et al.*, 2017; Kgopa *et al.*, 2018). There are many ways to get rid of this problem using chemical, physical and biological methods (Zhan and Ma, 2017).

The chemical and mechanical methods used to get rid of hydrocarbons from contaminated sites is expensive when the concentration level of the contaminants is high (Guru *et al.*, 2013). However, one promising method that has been generally used is the biological degradation of oil by microorganism since it will lead to mineralization with cost-effectiveness (Kumari and Amruta, 2013). Microorganisms were found as one of the most potent tools for remediation of crude oil (Pandey and Chandra, 2013). Bioremediation of contamination materials containing complex hydrocarbons is dependent on the capability of the bacteria or fungi to increase their population and colonies on these media and to biodegrade them to non-toxic products such as CO_2 and H_2O. Many environmental parameters affect the biodegradation efficiency of microorganisms, such as carbon and nitrogen concentration, temperature, oxygen availability, and pH (Ruffini *et al.*, 2016).

Biodegradation could be determined by many methods, including Gas chromatography technique which is used to determine the hydrocarbon degradation efficiency. It provides an exact and quick analysis that helps in the determination of the rate of biodegradation (Singh *et al.*, 2015). GC-MS is used to identify

[2]Department of Biology, College of Science for Women, Baghdad University, Baghdad, Iraq.
[3]Environment Research Centre, University of Technology, Baghdad, Iraq.
[4]National Research Center -Pests & Plant Protection Dep. Agriculture Division, Egypt.

all the composition of petroleum hydrocarbons. These methods are highly selective, and compounds can be authenticated by analyzing their unique mass spectra and retention times. GC-MS can prove the presence of target components and can be utilized for the separation of complex and simple hydrocarbons into groups. The aim of this study was to evaluate the efficiency of *Bacillus megaterium* in crude oil degradation, followed by the analysis of the biodegradation products using GC and GC-MS.

MATERIALS AND METHODS

The present study was conducted during March 2019 to January 2020 at the Directorate of Environment and Water, Baghdad, Iraq to study the bacterial biodegradation activity using Gas-Chromatography and Gas Chromatography-Mass Spectrometry.

Mineral Salt Medium (MSM)

The bacterial colonies were grown in Mineral Salt Medium (MSM) with the composition: $FeCl_3$ (0.002 g), $(NH_4)_2SO_4$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), KH_2PO_4 (1 g), $CaCl_2 \cdot 2H_2O$ (0.02 g), NaCl (1 g), Na_2HPO_4 (1 g), NH_4NO_3 (0.5 g), $MnSO_4 \cdot 2H_2O$ (0.002 g). These components were dissolved in 1L of distilled water (DW) of pH 7 and autoclaved for 15 min at 121°C (Herman *et al.*, 1997).

Modified Mineral Salt Medium (MMSM)

The MMSM was used in this study to facilitate the process of oil emulsion formation that will speed up the biodegradation process with the composition: $CaCl_2 \cdot 2H_2O$ (0.01 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), KH_2PO_4 (4g), NH_4NO_3 (4 g). These components were also dissolved in DW (1L, pH 7) and autoclaved for 15 min at 121°C (Duvnjak *et al.*, 1982).

Lactose-Broth

The purpose of this media is for the activation of the bacterial spores composed of yeast extract (5 g), NaCl (5 g), Glucose (1 g), Tryptone (10 g). These constituents were dissolved in 1L of DW (pH 7.2). From this solution, 26 mL was transferred to glass containers and autoclaved for 15 min at 121°C (Ball and Mccarthy, 1989).

Isolation and Identification of Bacteria from Soil Samples

The bacteria strains were isolated from the soil samples by a serial dilution technique. The selected colonies were identified via cultural, morphological and biochemical characteristics (Aneja, 2002).

Morphological Changes Associated with Crude Oil Biodegradation

The bacteria (*B. megaterium*) utilizes the MSM for growth. 50 mL of MSM was transferred into glass flasks (4 replicates) and sterilized for 15 min at 121°C. Then, 2 mL each of crude oil and the bacterial broth culture was added into the sterilized MSM and incubated for 1 week at 31°C (Kosaric, 2001).

GC and GC-MS-Assisted Detection of Crude Oil Degradation Products

The bacterial isolate, already grown in Lactose-Broth Medium for 24hr was seeded into a flask containing 50 mL of MSM, followed by the addition of 2 mL of crude oil into the flask (4 replicates). Then, the flasks were incubated at 31°C for 1 week in a rotary shaker incubated at 150 RPM. After the incubation period, the content of the flasks was centrifuged at 9,000 RPM for 1hr to obtain the cell precipitates. The filtrate obtained was resuspended 1:1 in hexane and dried in a rotary evaporator at 80°C. The dried filtrate, which represents the hydrocarbons residue, was analyzed in GC device (model: Shimadzu Japanese Company, 2014) using the CPSIL5-CB capillary column. The amount of hydrocarbon residues after microbial degradation was calculated from the resulted areas using the following equation (Kates, 1972):

$$\text{Area} = \frac{1}{2} \text{ base x height}$$

$$\text{Remaining percentage of hydrocarbons} = \frac{\text{Sample area}}{\text{total area}} \times 100$$

RESULTS AND DISCUSSION

Morphological Changes Associated with Crude Oil Biodegradation

From the results of the study, surface changes were observed in the crude oil layer due to the bacterial growth after 1 week of incubation at 31°C. There was a shift in the mass of the crude oil layer to the gelatinous bloc, accompanied by decrease in strength and emulsification compared to control (Plate 1). This observation points towards the bacterial capability to produce bio-emulsions which facilitated the biodegradation of crude oil (Al-Jubouri, 2004; Nwaogu *et al.*, 2008). In the gelatinous form, the mass of crude oil is described as pseudo-solubilization; it is the manifestation of the accumulation of emulsifying agents which reduces the oil to small droplets, causing their spreading and formation of emulsion (Plate 1). As per Kosaric (2001), the characteristics of these materials include degradability, alteration of surface-active processes, generally low toxicity (such as wetting and penetrating actions), reduction of surface and interfacial tensions, spreading, hydrophobicity and hydrophilicity actions, microbial growth, antimicrobial action, and metal sequestration.

a b

Plate 1. Morphological changes observed in crude oil incubated with *Bacillus megaterium* for 1 week at 31°C on MSM broth: (a) control sample; (b) sample incubated with *Bacillus megaterium.*

GC and GC-MS-Assisted Detection of Crude Oil Degradation Products

Using the GC device, 43 chemical compounds were identified in the control sample (Fig. 1) while most of these compounds were obviously missing in the biodegraded sample as evidenced by the disappearance of their respective peaks. In the treated sample (Fig. 1), only 20 compounds were identified; the peak area of some of the chemical compounds also decreases when matched with the untreated sample (Figs. 1 and 2). This is clear evidence of the capability of *B. megaterium* to decompose some of the chemical components of crude oil. The adopted method provided a clear understanding of the specific compounds that are being degraded (Diaz *et al.*, 2002), as well as clarified the manner these compounds are being degraded in crude oil (Gilbert *et al.*, 2001; Sharma and Rehman, 2009).

The microorganisms involved in oil biodegradation are widely distributed in the environment and have been isolated from water and soil ecosystems. With their oil biodegrading capacity, the microorganisms can utilize oil products as a source of energy and carbon (Atuanya and Tudararo-Aherobo, 2014).

This study was performed to study the biodegradation of crude oil by *B. megaterium.* Chemical tests were performed using GC analysis; the treated samples were compared to the untreated samples. The results showed the presence of 43 compounds in the untreated crude oil while most of these peaks disappeared in the treated crude oil. Only 20 peaks were identified in the treated sample while the area of the other peaks was reduced, indicating the efficiency of *B. megaterium* in degrading hydrocarbon compounds (Fig. 2).

From the quantitative and qualitative analysis using GC-MS technique, some of the complex compounds that had been completely degraded include Butanoic acid, 1-1-dimethylethyl ester, Benz [c] pyran-1,3-dione, 4,4-dimethyl, 1,2-Benezenedicarboxylic acid, dioctyl ester, etc. the available polycyclic aromatic hydrocarbons (PAHs) were used by the microorganisms as carbon source for growth and replication. The microorganisms degraded each PAHs gradually to ensure their complete degradation (Figs. 3 and 4; Table 1). This indicated that the amounts of n-alkanes (lower molecular weight) dramatically decreased, and the bacteria and fungi required some enzymatic modifications to utilize the higher molecular weight hydrocarbons. On the other hand, another explanation for the

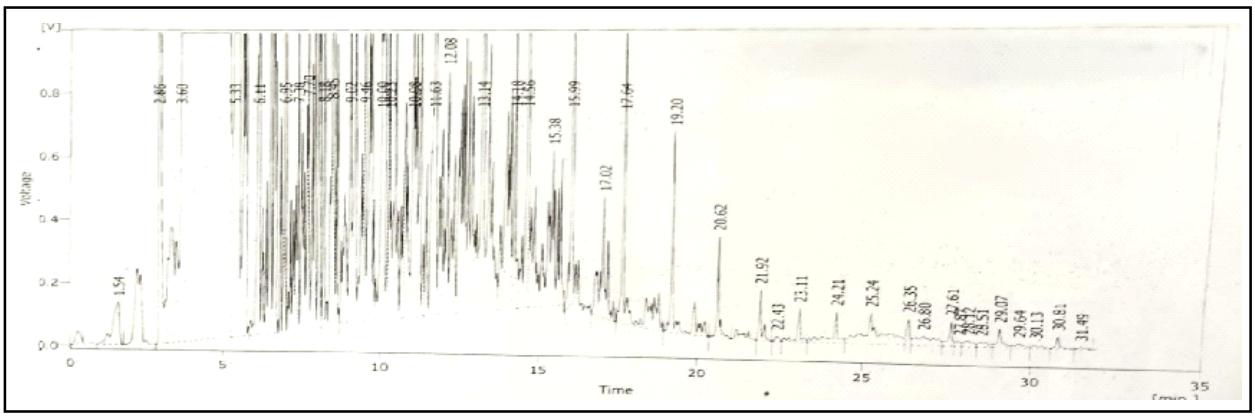


Fig. 1. Gas Chromatography (GC) for the control sample (untreated crude oil).

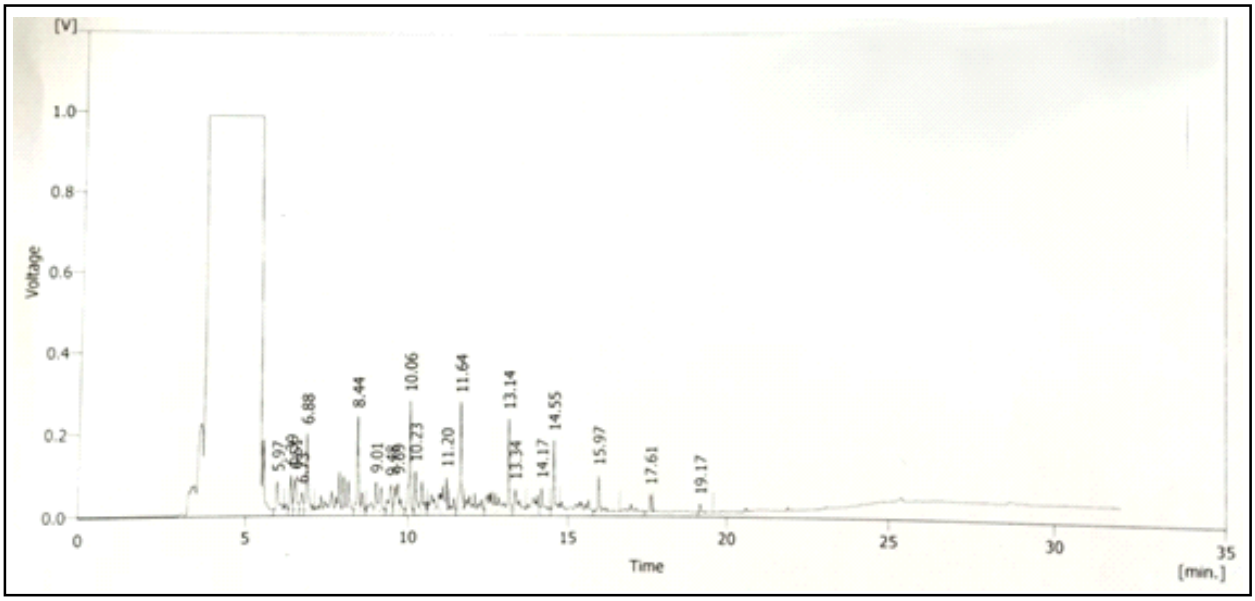


Fig. 2. Gas-Chromatography (GC) for a crude oil sample treated with *Bacillus megaterium*.

lateness in biodegradation was that the microorganisms may not prefer to degrade the high molecular weight hydrocarbons until the low molecular weight counterparts have been completely degraded (Malik and Safia, 2012).

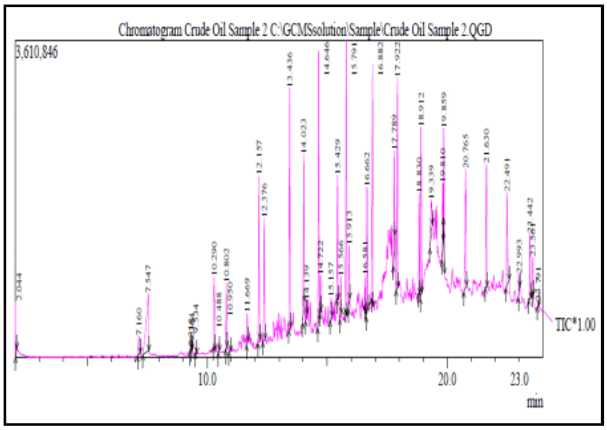
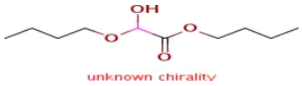
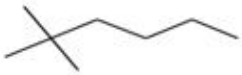
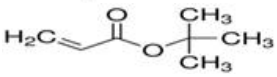
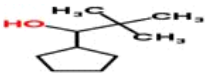
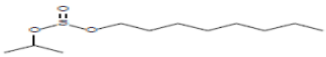
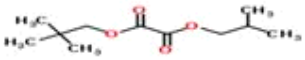
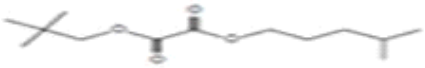
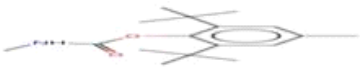
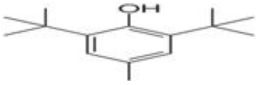


Fig. 3. Gas-Mass Chromatography (GCMS) for the control sample (untreated crude oil).

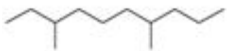
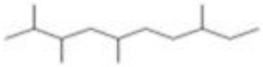
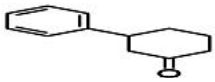
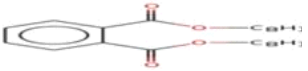
The results also showed that the bacteria can use both the long chain and short chain PAHs as a carbon source (Singh *et al.*, 2015). The biodegradation process of crude oil by several strains of bacteria, namely, *Alcaligenes sp. ASS-1, Alcaligenes sp.* ASW-3, *Pseudomonas aeruginosa strain ASW-2, Exiguobacterium sp. ASW-1,* and *Bacillus sp. ASS-2* was evaluated using GC-MS in previous studies (Santhakumar *et al.*, 2017; Chen *et al.*, 2017). The Determination of crude oil degradation efficiency via GC-MS revealed that the most efficient strain is *Serratia proteamaculans S1BD1*, followed by *Alcaligenes sp. OPKDS2* and *Rhodococcus erythropolis OSDS1* (Xia *et al.*, 2017). Additionally, *Microbacterium hydrocarbonoxydans* showed

Table 1. The hydrocarbons compound degraded by the action of B. megaterium through the results of the gas-mass chromatography

Compound name	Chemical structure
Butanoic acid,1-1-dimethylethyl ester	
Benzene, (2-methyloctyl)	
Boron, (3,3-dimethylbutyl) (n-methylenemethan amine)	
Benzeneacetic acid, heptyl ester	
Oxalic acid, butyl propyl ester	
Propane,1-bromo-2,2-dimethyl	
Acetic acid, butoxyhydroxy, butyl ester	
Hexan, 2-dimethyl	
Tert-butyl acrylate	
1-cyclopentyl1-2, 2-dimethyl-1-propanol	
Sulfurous acid, octyl 2-propyl ester	
Nonane, 3,7-dimethyl	
Oxalic acid, isohexylneopentyl ester	
Sulfurous acid, nonyl 2-propyl ester	
Sulfurous acid, decyl 2-propyl ester	
Nonane, 3, 7-dimethyl	
Oxalic acid, isohexylneopentyl ester	
Phenol,2,6-bis(1,1-dimethylethyl)-4-methyl, methylcarbamate	
4,6-di-tert-butyl-m-cresol	
Butylated hydroxytoluene	

Contd.

Table 1 cond.

Compound name	Chemical structure
Cyclopropanecarboxylic acid,1-hydroxy	
Phenol,4-6di(1,1dimethylethyl)2-methyl	
Decane,3,7-dimethyl	
Decane,2,3,5,8-tetramethyl	
Undecane ,3-ethyl	
Octane,3,4,5,6-tetramethyl	
Undecane,3,8-dimethyl	
Benz [c] pyran-1,3-dione, 4,4-dimethyl	
3-phenylthiane,s,s-dioxide	
1,2,4-metheno-1H-cyclobuta[cd]pentalene-3,5-diol,octahydro	
Cyclohexanone, 3-phenyl	
1,2-Benzenedicarboxylic acid	
1,2-Benezenedicarboxylic acid, diisooctyl ester	
Bis(2-ethylhexyl) phthalate	

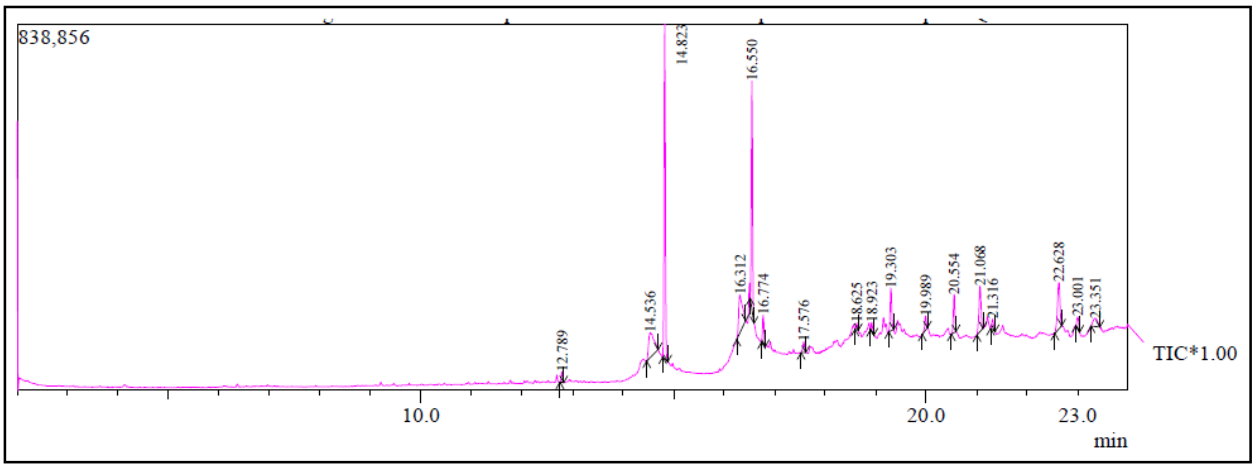


Fig. 4. Gas-Mass Chromatography (GCMS) for a crude oil sample treated with *Bacillus megaterium*.

high crude oil-degrading capability as exhibited by its high growth rate in crude oil-enriched medium (Santhakumar *et al.*, 2017).

CONCLUSION

Bacillus megaterium isolate under laboratory conditions demonstrated the capability in crude oil degradation by using the crude oil components as carbon sources for growth. *B. megaterium* exhibited the highest level of oil degradation and had the best development processes because this experiment mimicked the natural ecosystem where the organism thrives. This work is significant as it can guide further biodegradation and remediation studies, as well as provide useful information that will guide the use of other bacteria for biodegradation processes in crude-oil contaminated environments. However, there is a need to conduct more studies on the characterization of the bio-emulsion produced by *B. megaterium* and its impact on the ecosystem.

REFERENCES

Al-Jubouri, M. K. S. (2004). Role of some cyanobacterial species in biodegradation of some petroleum compounds. M. S. Thesis, Tikrit University, Tikrit, Iran.

Aneja, K. R. (2002). Experiments in microbiology, plant pathology, tissue culture and mushroom production technology, 4th Edn. New Age International (P) Ltd., New Delhi. Pp.161-62.

Atuanya, E. and Tudararo-Aherobo, L. (2014). Ecotoxicological effects of discharge of Nigerian petroleum refinery oily sludge on the biological sentinels. *Afr. J. Environ. Sci. Technol.* **9** : 95-103.

Ball, A. S. and Mccarthy, A. J. (1989). Production and properties of xylanases from actinomycetes. *J. Appl. Bacteriol.* **66** : 439-44.

Chen, Q., Li, J., Liu, M., Sun, H. and Bao, M. (2017). Study on the biodegradation of crude oil by free and immobilized bacterial consortium in marine environment. *Plots ONE* **12** : Article No. e0174445. doi.org/10.1371/ journal.pone.0174445.

Diaz, M. P., Boyd, K. G., Grigson, S. J. W. and Burgess, J. G. (2002). Biodegradation of crude oil across a wide range of salinities by an extremely halotolerant bacterial consortium MPD-M, immobilized onto polypropylene fibers. *Biotechnol. Bioeng.* **79** : 145-53.

Duvnjak, Z., Cooper, D. G. and Kosaric, N. (1982). Production of surfactant by *Arthrobacter paraffineus* ATCC 19558. *J. Biotechnol. Bioeng.* **24** : 165-75.

Gilbert, F., Stora, G., Desrosiers, G., Deflandre, B., Bertrand, J. C., Poggiale J. C. and Gange J. P. (2001). Alternation and release of aliphatic compounds by the polychaete *Nereis virens* (Sars) experimentally fed with hydrocarbons. *J. Exp. Mar. Biol. Ecol.* **256** : 199-13.

Guru, G., Gohel, H., Ghosh, S. and Braganza, V. (2013). Isolation and enrichment of microbes for degradation of crude oil. *Int. J. Eng. Sci. Innov. Technol.* **2** : 144-47.

Herman, D. C., Zhang, Y. and Miller, R. M. (1997). Rhamnolipid (biosurfactant) effect on cell agreement and biodegradation of residual hexadecane under saturated flow conditions. *J. Appl. Environ. Microbiol.* **63** : 3622-67.

Kates, M. (1972). Techniques of lipidology. In: Work, T. S. and Work, E. (Eds.), Techniques of Lipidology: Isolation, Analysis and Identification of Lipids, American Elsvier Publishing, Co., Inc. New York.

Kgopa, P. M., Mashela, P. W. and Manyevere, A. (2018). Accumulation of heavy metal in onion (*Allium cepa*) plants irrigated with treated wastewater under field conditions. *Res. Crops* **19** : 62-67.

Kosaric, N. (2001). Biosurfactants and their application for soil bioremediation. *Food Technol. Biotech.* **39** : 259-04.

Kumari, S. P. and Amruta, S. R. (2013). Analysis of biodegradation pathway of crude oil by *Pseudomonas* sp. isolated from marine water sample. *Arc. Appl. Sci. Res.* **5** : 165-71.

Malik, Z. A. and Safia, A. (2012). Degradation of petroleum hydrocarbons by oil field isolated bacterial consortium. *Afr. J. Biotech.* **11** : 650-58.

Misra, V. and Pandey, S. D. (2005). Hazardous waste, impact on health and environment for development of better waste management strategies in future in India. *Environ. Int.* **31** : 417-31.

Nwaogu, L. A., Onyeze, G. O. and Nwabueze, R. N. (2008). Degradation of diesel oil in a polluted soil using *Bacillus subtilis*. *Afr. J. Biotech.* **7** : 1939-43.

Pandey, A. and Chandra, R. (2013). Isolation of oil degrading bacteria from oil contaminated soil and expression of oil degrading genes in non-oil degrading bacteria. *J. Drug Discov. Ther.* **1** : 1-17.

Rajput, S. G., Towari, D. D., Katiyar, N. K., Kumar, A., Pathak, R. K. and Srivastava, V. (2017).

Effect of industrial effluent on soil properties, essential nutrient and pollutant element status of soils and plants in a vegetable growing near Jajmau industrial area of Kanpur, Uttar Pradesh, India. *Res. Crops* **18** : 249-55.

Ruffini, C. M., Giorgetti, L., Becarelli, S., Siracusa, G., Lorenzi, R. and Di Gregorio, S. (2016). Polycyclic aromatic hydrocarbon-contaminated soils: bioaug-mentation of autochthonous bacteria and toxicological assessment of the bioremediation process by means of *Vicia faba* L. *Environ. Sci. Pollut. R.* **23** : 7930-41.

Santhakumar, M., Sivalingam, S., Dharmapal, D., Sadras, S. R. (2017). Characterization of dioxygenases and biosurfactants produced by crude oil degrading soil bacteria. *J. Microbiol.* **48** : 637-47.

Sharma, A. and Rehman, M. B. (2009). Laboratory scale bioremediation of diesel hydrocarbon in soil by indigenous bacterial consortium. *Indian J. Exp. Biol.* **47** : 766-69.

Singh, P., Dipali, P. and Ajit, P. (2015). Comparative study of crude oil degradation efficiency of microbes isolated from crude oil contaminated site. *Bull. Environ. Pharma. Life Sci.* **4** : 91-94.

Xia, M., Yi Liu, A. A. T., Dafang, F., Abdur, R. K. and Norman, T. (2017) Crude oil depletion by bacterial strains isolated from a petroleum hydrocarbon impacted solid waste management site in California. *Int. Biodeterior. Biodegradation* **123 :** 70-77.

Zhan, Y. B. and Ma, L. A. (2017). Research progress on bioremediation of petroleum contaminated soil. *J. Yangtze University* **13** : 52-56.